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Molecular Tools for Understanding the Population Genetic Effects of Habitat Restoration on Butterflies

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MOLECULAR TOOLS FOR UNDERSTANDING THE POPULATION GENETIC EFFECTS OF HABITAT RESTORATION ON BUTTERFLIES

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By

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ABSTRACT

The EPA funded Upper Green River Conservation Reserve Enhancement Program (CREP) pays farmers to plant native prairie plants in the place of agricultural crops and to restrict cattle grazing along the river. One of the expected effects of this change in land use is that it will create habitat corridors between isolated patches of suitable habitat for many species of butterflies. The construction of such corridors is predicted to increase the connectedness among populations of butterfly species whose larval host plants are either included in the native grass seed mixes (*Everes comyntas*, *Chlosyne nycteis, Phoebis sennae,* and *Phyciodes tharos*) or whose host plants are damaged by grazing *(Pterourus troilus*). To test for this effect, we have collected samples of these five species plus one control species (*Pterourus glaucus*) not expected to be influenced by the CREP manipulation, from eight sites along the Upper Green River CREP district in south central Kentucky. DNA was isolated from legs of each specimen. The population structure data comes from the analysis of Randomly Amplified Fingerprints (RAF), a technique for studying genetic variation that is highly repeatable and easily scored on an automated fluorescent DNA sequencer. By collecting data in the early stages of CREP, we can establish a baseline to compare to future data obtained after the CREP program has matured and determine the efficacy of the program on reuniting previously isolated butterfly populations.

Keywords: Population Genetics, Genetic Fingerprinting, Butterfly Conservation

Dedicated to my fiancé Katie, my friends and my family

ACKNOWLEDGMENTS

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INTRODUCTION

Habitat isolation has important effects on the structure of butterfly populations (Forister et al. 2004; Rogo and Odulaja 2001; Thomas et al. 1998; Williams et al. 2003; Zakharov and Hellman 2008). These effects can be linked to both natural and anthropogenic causes. One observed effect is that populations that are isolated by fragmentation will begin to differ genetically (Williams et al. 2003). The extent of this variation in fragmented populations lies principally with the ability of the species to cope with habitat variability, for example if the species is known to be a habitat specialist or generalist (Krauss et al. 2003). Many believe that by conserving existing areas of butterfly habitat and replanting larval host plants, the genetic effects of population sub-division may subside (Krauss et al. 2005; Kronforst and Fleming 2001).

Due to the intensive agricultural use of the land in South Central Kentucky, many native plant species have become rare or locally extirpated (Kentucky State Nature Preserves Commission 2005). This has caused several butterfly species, which depend on these plants as larval hosts, to also become less abundant (Covell 1999). The Conservation Reserve Enhancement Program (CREP) was established by the US Environmental Protection Agency to provide incentives for farmers to commit to 10 or 15 year Conservation Easements on their properties with state and federal government in order to establish wildlife habitat corridors and protect water quality. The

Commonwealth of Kentucky agreed on August 29, 2001 to take part in this voluntary program to establish a CREP habitat corridor along the Upper Green River which runs through South Central Kentucky. One of the anticipated effects of creating this corridor is that it will reunite previously fragmented populations of butterflies and other species that live along the corridor.

The Green River watershed is of particular importance due to its high aquatic biodiversity (Thomas 2003), but its terrestrial diversity is less well studied. Butterfly populations in the Green River watershed are used in this study to explore the effects of habitat subdivision on species with different habitat requirements. Three categories of species are included. First, are species that as larvae feed directly on native plants that are included in the CREP seed mixes (Table 1). Species in this group include the silvery checkerspot butterfly (*Chlosyne nycteis*, feeds on sunflowers and other composites), the eastern tailed-blue butterfly (*Everes comyntas,* feeds on native peas and other legumes), the cloudless sulfur (*Phoebis sennae,* feeds on partridge pea), and pearl crescents (*Phyciodes tharos*, feeds on asters) (Glassberg 1999; Brock and Kaufman 2003; Cech and Tudor 2005). Planting native host plants for these species in the habitat corridors are expected to enlarge the habitat areas suitable for these species and increase the connectedness between extant populations.

The second category includes a butterfly species whose larvae feed on plants that are not included in the CREP seed mix, but which are expected to increase in abundance due to the removal of the grazing animals. The larvae of this species, the spicebush swallowtail (*Pterorous troilus*) feed on spicebush host plants (*Lindera benzoin*) that are not eaten by cattle, but which do not grow well in soil compressed and disturbed by cattle

(Recce 1986). By removing cattle grazing from the habitat corridor, the host plants and the butterflies that depend on them should become more abundant, with likely positive effects on migration rates and connectedness between habitat patches. The final category includes a species that is not expected to be affected by the creation of the habitat corridor because its larvae feed on host plants that are not included in the CREP seed mix and whose population structure is not expected to be greatly affected by changes in grazing practice. Included in this category are tiger swallowtails (*Pterorous glaucus*) which feed on tulip poplar. By analyzing the population structures of these species of butterflies early in CREP development and later once the project has been in effect for several years, this study hopes to provide an evaluation as to the efficacy of the program.

To study the population structures of these butterflies, a technique known as Randomly Amplified Fingerprints (RAF) was used (Schlipalius et al. 2001; Waldron et al. 2002). RAF is a technique that characterizes genetic variation and is very similar to Randomly Amplified Polymorphic DNA (RAPD). Some key differences between the two are that RAF is highly repeatable and is easily scored on a fluorescent automated DNA sequencer (Marcus et al. submitted). This fingerprinting technique will be used to obtain the data to make phylogenetic trees to determine the relatedness between and among the populations. We anticipate that species likely to benefit from CREP will show higher levels of tree structures initially and will lose structure as CREP becomes implemented for longer periods of time and populations have become more inter-connected.

METHODS AND MATERIALS

Sample Collection

Butterfly species included in this study were selected using the criteria of availability throughout the sampling region, sufficient abundance, and to include species that were expected to respond differently to the CREP manipulation. Samples of each species were collected from 8 suitable habitat locations along the Green River in South Central Kentucky including sites in Hart, Green, and Taylor Counties (Figure 1, Table 2). The straight-line distance between the two most distant sampling locations (the Upper Green River Biological Preserve and Tebbs Bend) is 55.7 km and the locations are approximately 105.7 "river kilometers" apart following the course of the Green River. Sampling sites were generally adjacent to the Green River and included river banks where butterflies congregated in mud-puddling aggregations and adjacent fields where they were found nectaring. Collection localities were visited weekly from June to September 2008 and butterflies were captured with hand-held butterfly nets. I endeavored to obtain 10 samples of each species from each location (80 samples total for each species) to allow assessment of within-population genetic variation, but due to variations in abundance, the actual number of specimens studied from each species varied from 37 to 110 (Table 2). Voucher specimens from this study remain in the research collection of Dr. Jeffrey Marcus

DNA Isolation

DNA from each butterfly sample was isolated from the legs using a Qiagen DNeasy Blood and Tissue kit. Standard protocols provided by the kit for DNA extraction from insect tissues were followed with two exceptions: initially 1-2 legs from each specimen were crushed using mortar and pestle in ATL buffer prior to extraction and a smaller final elution volume of 100 µL divided in two 50 µL elutions was used to increase final DNA concentration.

Randomly Amplified Fragment (RAF) Preparation

Each sample was prepared for Randomly Amplified Fingerprinting (RAF) using 1 µL DNA template from the DNEasy extraction, 4 µLTaq polymerase master mix (Eppendorf or New England Biolabs) and 5 µL 6-Fam-conjugated RP2 fluorescent primer (Schlipalius et al., 2001) of a 1/10 dilution from the 100 µM primer stock solution. DNA from each specimen was amplified and analyzed in triplicate in parallel with a negative control of Nanopure^{TM} water and a positive control of DNA from an intensively studied butterfly specimen (e.g. *Limenitis arthemis astyanax* specimen RP3 *(Marcus et al., ms)*, *Junonia coenia* specimen TXC2, and *Everes comyntas* specimen UGRBP_EB1.1). RAF reactions were amplified in a BioRad Mycycler Thermocycler using the following program: 95˚C for 5 minutes; 30 cycles of 94˚C for 30 seconds, 57˚C for 1 minute, 56˚C for 1 minute, 55˚C for 1 minute, 54˚C for 1 minute, and 53˚C for 1 minute; followed by a 5 minute extension at 72˚C and a final holding temperature of 4˚C (Marcus et al. submitted).

After amplification, 10 μL HiDye formamide (Applied Biosystems) and 1 μL ROX-500 GeneScan Size Standard (Applied Biosystems) was added to each PCR tube. The resulting solution was mixed by vortexing for 1-2 seconds and put in a microcentrifuge at 13,000 rpm for 30 sec. Each sample was then removed from the PCR tubes and placed into individual wells on a sequencing plate. This plate was then run on a 95˚C cycle for 4 minutes and then put on ice for 3-5 minutes before being loaded into an ABI 3130 Capillary Sequencer fitted with a 50 cm capillary array and filled with POP-7 polymer.

Analysis of RAF Results

The samples were analyzed by an ABI automated sequencer in conjunction with GENEMAPPER version 3.7 software (Applied Biosystems). Using the software, an allelic bin size of 3 was chosen for study due to its ability to detect polymorphic alleles without introducing excessive noise in the data associated with small differences in run time between samples. The resulting Genemapper genotype data was exported to an Excel spreadsheet for further analysis. Bands that appeared in negative control amplifications were considered artifact and removed from further analysis for all samples. Within the 3 replicate samples from an individual butterfly, allele calling for each allele was based on a majority rule basis, and each allele was coded in binary, with 0 indicating the absence of an allele and 1 indicating the presence of the allele. This binary data was then analyzed using the Neighbor-Joining distance settings of PAUP* (Swofford 1998) phylogenetic analysis software to generate trees showing the genetic

relatedness of each of the samples within a species. The outgroups used for the tree generation were the positive control specimens.

RESULTS

RAF results for each of the species include the total number of alleles coded and informative alleles. Informative alleles include those that are both variable within the species and shared by at least two individuals of the species. For *E. comyntas* there were 59 total alleles with 44 of those alleles being informative. *Pterorous troilus* showed 21 total alleles and 17 informative alleles. RAF data for *C. nycteis* showed 70 total alleles including 44 informative alleles. *Phoebis sennae* data gave 27 total alleles and 22 informative alleles. Analysis of the *P. glaucus* data resulted in 44 total alleles with 27 being informative. Finally, *P. tharos* data gave 170 total alleles and 165 informative alleles.

The Neighbor-Joining distance trees for each species generated by RAF analysis in the PAUP software show differing amounts of structure. To facilitate describing the observed relatedness among populations from the different sites, I have grouped the sampling localities into Western, Central and Eastern groups. The Western group includes a single locality: the Upper Green River Biological Preserve (UGRBP). The Central group includes the Thelma-Stovall Park in Munfordville (TSM) and Lynn Camp Creek (LCC) sites. The Eastern group includes Glenview Road (GVR), the Green River Paddle Trail (GRPT), American Legion Park (Green County, KY) (ALP), Roachville (RV), and Tebbs Bend (TBNA and TBB).

Analysis of data from two species: *E. comyntas* and *P. troilus* produced trees that revealed the most population structure with distinct clades for each of the three identified regions within the study area: West, Central, and East (Figures 2 and 3 respectfully). Two species' trees illustrate an intermediate amount of structure. The *C. nycteis* tree (Figure 4) has a distinct clade with only samples from Eastern sites followed by a large mixed clade. The *P. sennae* tree (Figure 5) has two clades that contain Central and Western sites along with an extensive Eastern site-dominated mixed clade. The final two species' trees, *P. glaucus* and *P. tharos* in Figures 6 and 7 respectfully, have only large mixed clades in their structure.

DISCUSSION

The variation in the amount of population structure observed in the six species studied here would have been very difficult to predict. Two strong flyers, the two largest species (both swallowtails in the family Papilionidae) , *P. glaucus* and *P. troilus*, show the smallest, and the largest amount of population structure, respectively. The two smallest species (which are relatively weak flyers), *P. tharos* and *E. comyntas*, show very little and extensive population structures, respectively. The two intermediately sized species, *C. nycteis* and *P. sennae*, both show intermediate levels of population structure.

Taxonomically, the two species of butterflies in the family Nymphalidae, *C. nycteis* and *P. tharos* show intermediate and low population structure. The single species in the family Lycaenidae, *E. comyntas*, shows extensive population structure, while the

single species in the family Pieridae, *P. sennae,* shows intermediate population structure. Finally, as mentioned previously, the two species in the family Papilionidae show very high and very low levels of population structure. Thus, I did not detect any taxonomic groups that tended to have consistently high or consistently low genetic population structure.

Host plant use was similarly unhelpful in predicting population structure. Larval host plant generalists (*E. comyntas*, *P. tharos*, and *C. nycteis*), which feed on multiple plant species show extensive to intermediate population structure. Larval host plant specialists (*P. glaucus, P. troilus, P. sennae)* ranged from highly structured to highly unstructured populations. Collectively, these results suggest that conducting pilot studies of genetic diversity is critical in order to determine which butterfly species have suitable initial amounts of population structure such that the expected effects of corridor construction can be evaluated. Further, at least for the species and geographic context considered in this study, the degree of larval host specialization is not a good predictor of the degree of population structure found in butterfly populations, contrary to the predictions of Krauss et al. (2003).

One of the key goals of this study was to identify butterfly species that show substantial population structure at the beginning of the CREP planting manipulation in the Upper Green River basis. One butterfly species that feeds on plants included in the CREP seed mix, *E. comyntas,* and one butterfly species whose larvae feed on a plant that is negatively impacted by grazing, *P. troilus,* show extensive population structure. Two additional species, *C. nycteis* and *P. sennae* with host plants included in the CREP seed mix also show some population structure. These four species are the best candidates for

future studies of butterfly population structure to detect changes as a result of the CREP manipulation. It is expected that once the seed plantings have matured and land use changes have been fully implemented in the Upper Green River watershed, habitat corridors will be established leading to gene flow between any fragmented populations, producing a decrease in the population structure of these butterfly species within this region.

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Table 1. Plant species included in seed mixes used in CREP plantings in the Upper Green River Corridor

Scientific name	**Common Name**
Andropogon gerardii	big bluestem grass
Bouteloua curtipendula	side oats grama grass
Chamaecrista nictitans	sensitive partidge pea
Dalea candida	white prairie clover
Dalea purpurea	purple prairie clover
Desmanthus illinoiensis	Illinois bundleflower
Echinacea purpurea	purple coneflower
Elymus virginicus	Virginia wild rye
Heliopsis helianthoides	false sunflower
Lespedeza capitata	round-headed bush clover
Liatris aspera	tall blazing star
Monarda fistulosa	bee balm
Panicum virgatum	switch grass
Ratibida pinnata	pinnate prairie coneflower
Rudbeckia hirta	black-eyed susan
Schizachyrium scoparium	little bluestem grass
Sorghastrum nutans	Indian grass
Symphyotrichum novae-angliae	New England aster
Tripsacum dactyloides	Eastern gama grass

Table 2. Butterfly specimen collection localities and sample sizes.

Location	**GPS**	*Pterorous glaucus*	*Pterorous troilus*	*Phoebis sennae*	*Everes comyntas*	*Phyciodes tharos*	*Chlosyne nycteis*	**Totals**
Upper Green River Biological Preserve (UGRBP)	37° 14.255', 085° 59.552'	7	14	10	13	13	10	67
Thelma-Stovall Park, Munfordville (TSM)	37° 15.998', 085° 53.328'	12	10	13	12	4	7	58
Lynn Camp Creek (LCC)	37° 19.035', 085° 46.096'	5	6	3	12	6	2	34
Glenview Road (GVR)	37° 17.468', 085° 35.472'	1	1	11	13	9	1	36
Green River Paddle Trail (GRPT)	37° 15.490', 085° 30.336'	6	1	10	13	3	9	42
American Legion Park in Green County, KY (ALP)	37° 14.653', 085° 28.788'	7	4	10	14	11	8	54
Roachville (RV)	37° 14.116', 085° 25.477'	4	1	12	12	3	10	42
Tebbs Bend (TBB & TBNA)	37° 14.744', 085° 21.919'	1	0	1	21	3	4	30
	Total	43	37	70	110	52	51	363

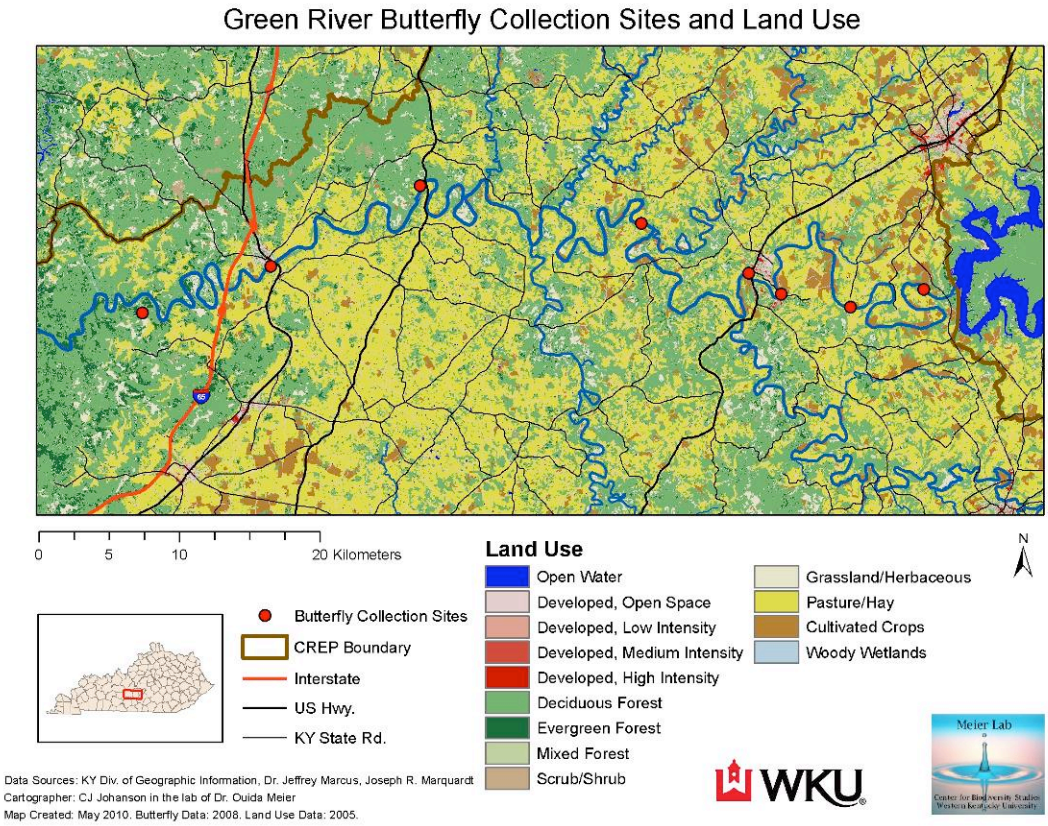

Figure 1 Map of Upper Green River corridor in which sampling took place. Red dots indicate sampling localities. From West to East, the sampling localities were the Upper Green River Biological Preserve in the Western grouping; Thelma-Stovall Park in Munfordville and Lynn Camp Creek in the Central grouping; and Glenview Road, Green River Paddle Trail, American Legion Park in Green County, KY, Roachville, and Tebbs Bend in the Eastern grouping.

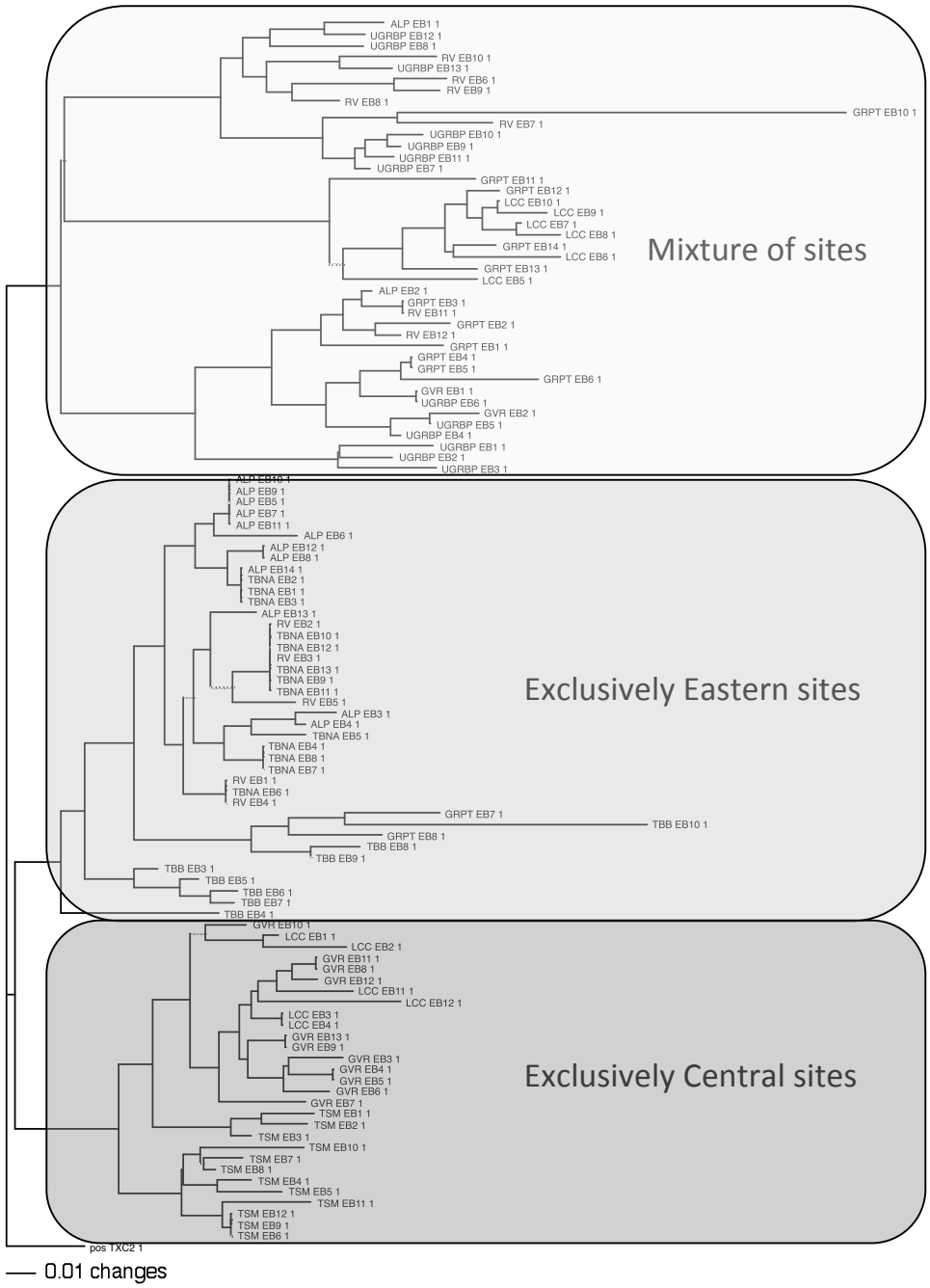

Figure 2 Distance tree for *Everes comyntas*.

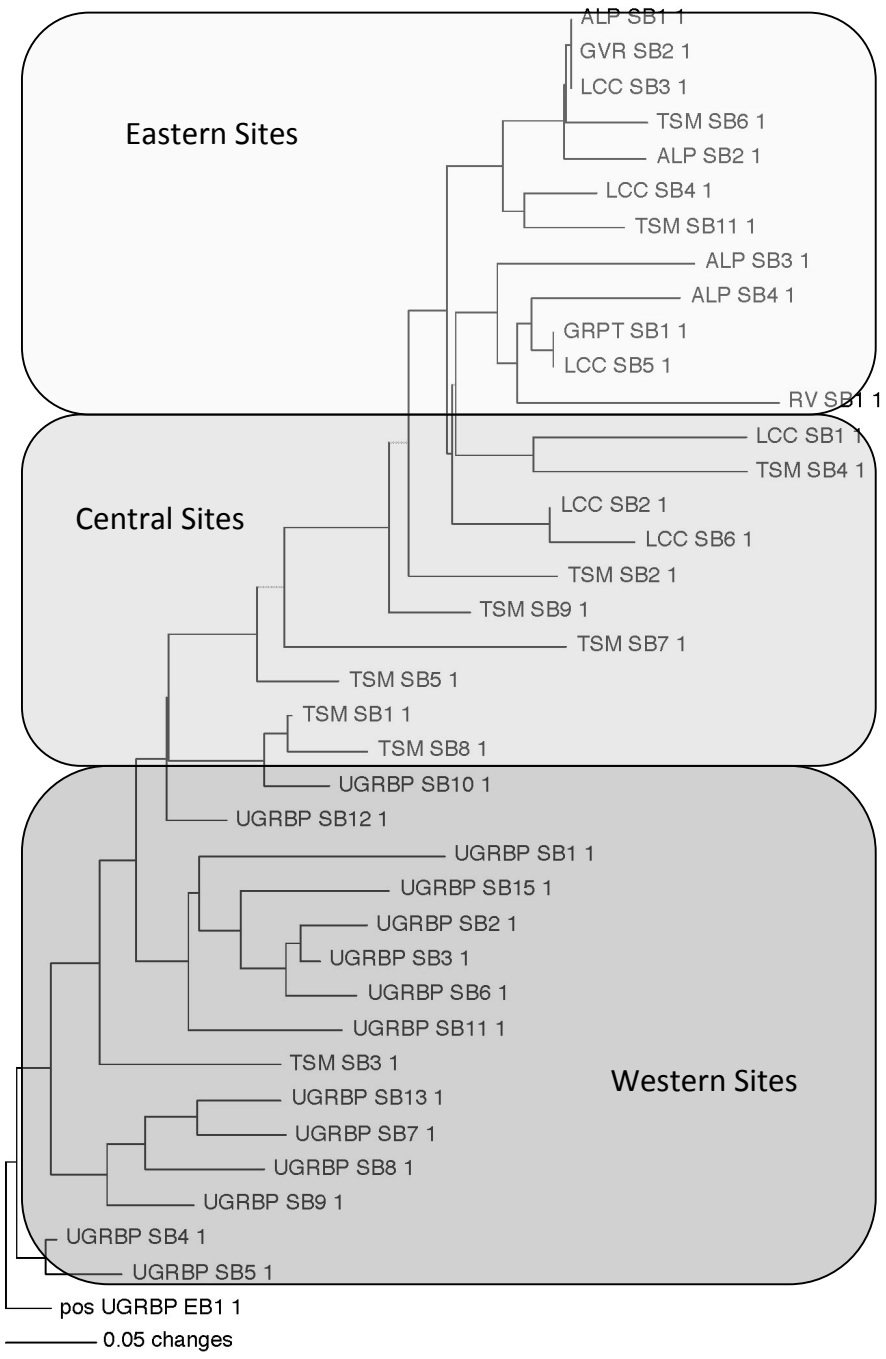

Figure 3 Distance tree for *Pterorous troilus*

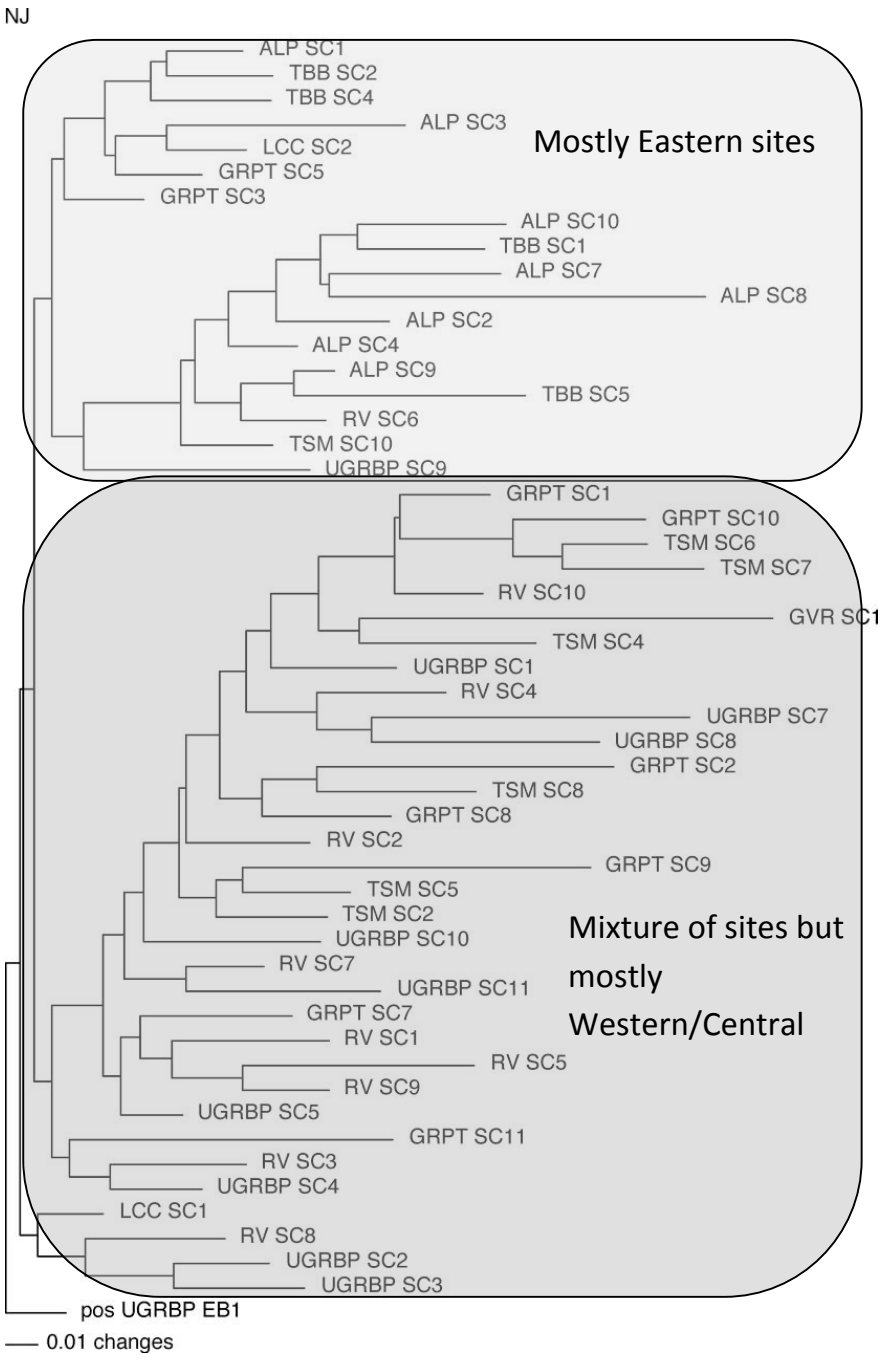

Figure 4 Distance tree for *Chlosyne nycteis*.

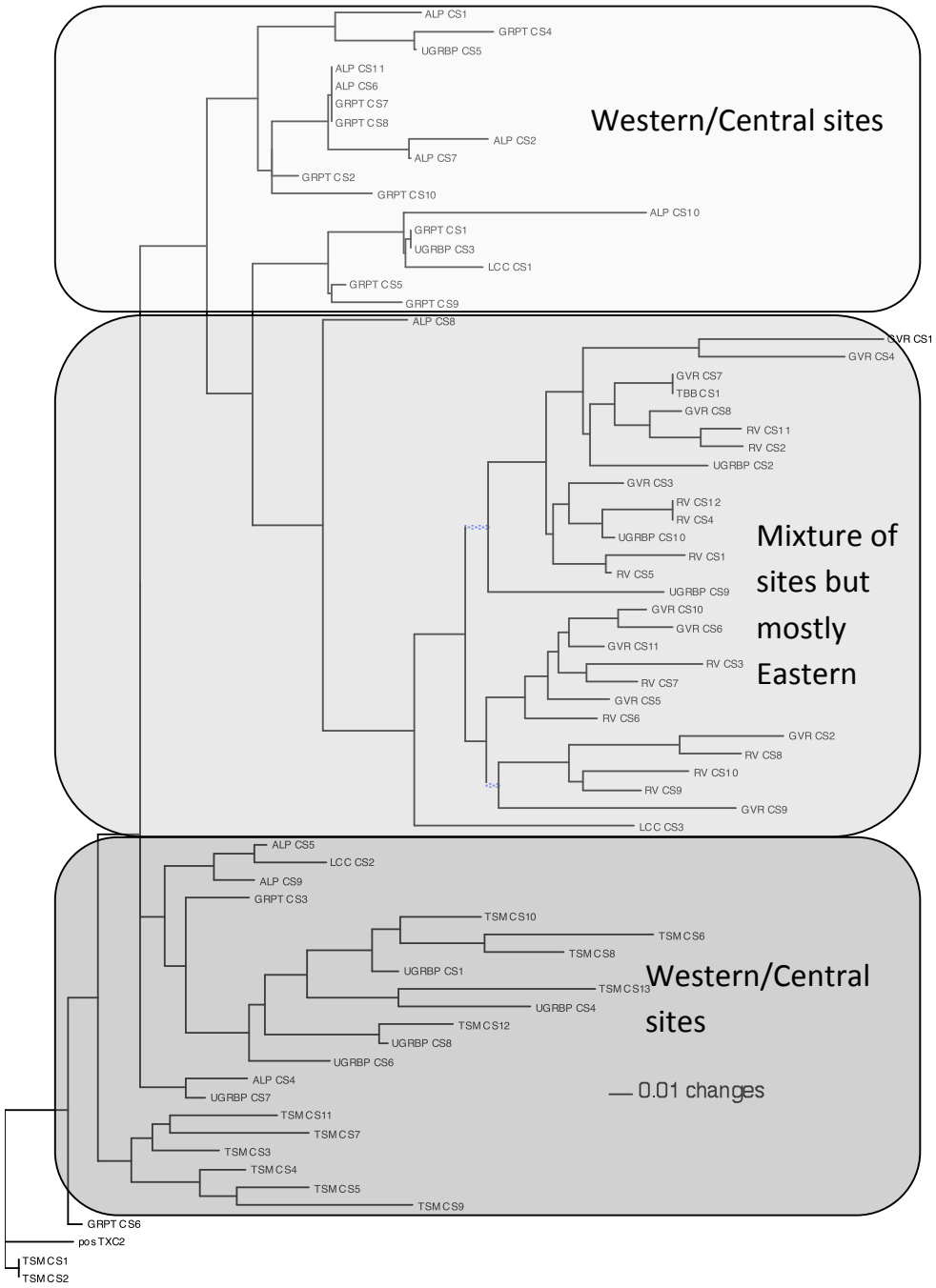

Figure 5 Distance tree for *Phoebis sennae*.

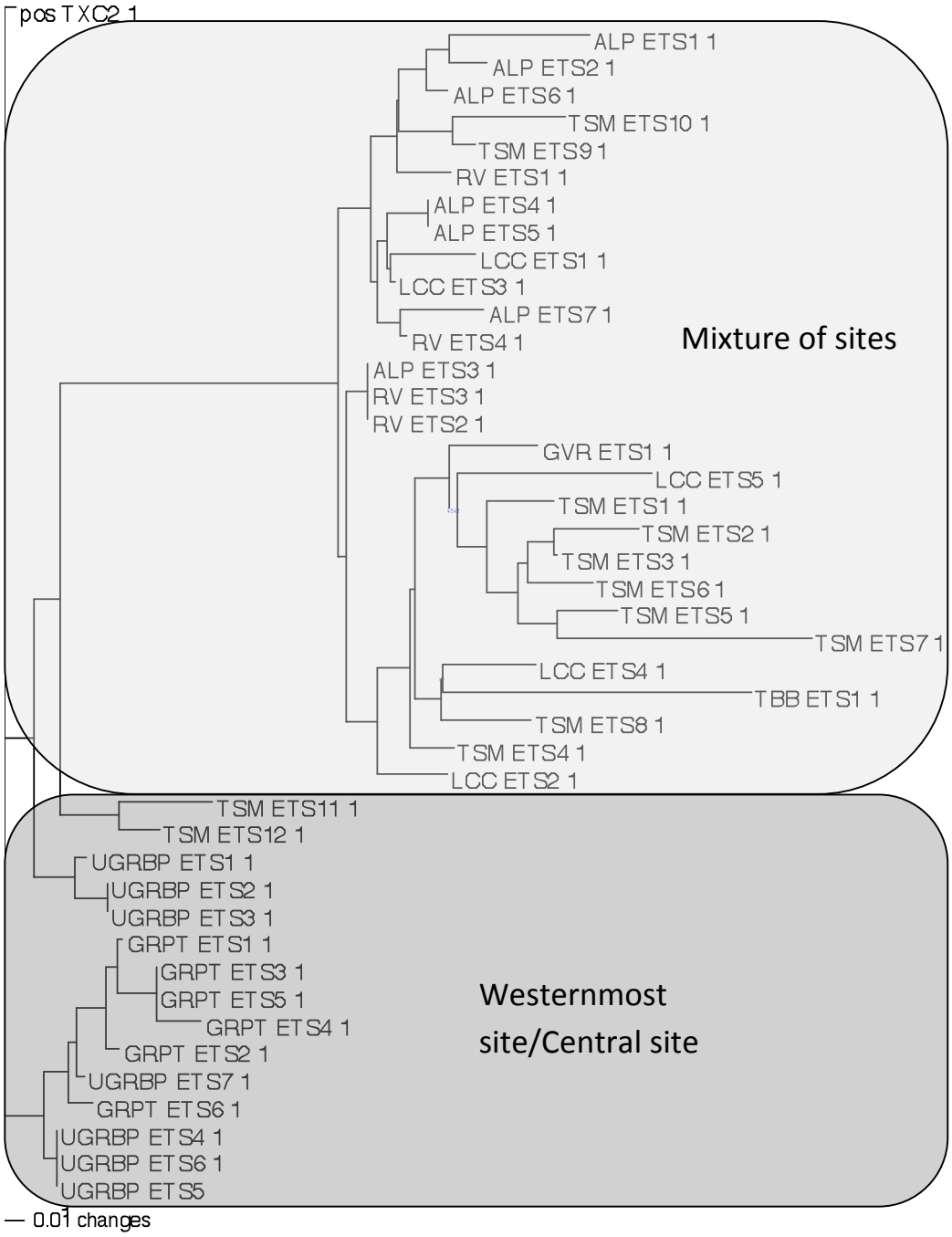

Figure 6 Distance tree for *Pterorous glaucus*.

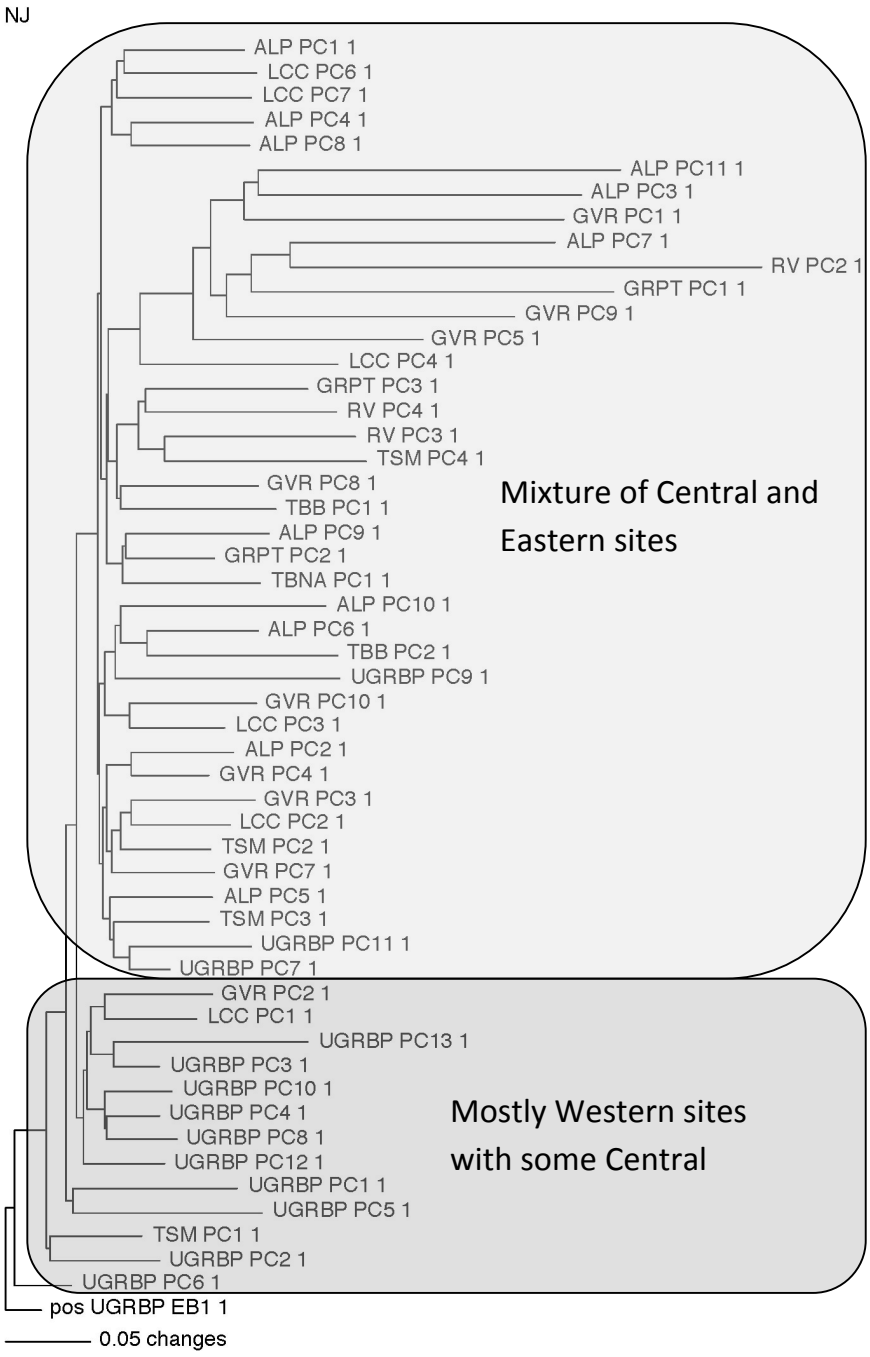

Figure 7 Distance tree for *Phyciodes tharos*.